FINAL REPORT

Facilitated Immobilization of Heavy Metals in Soil by Manipulation with Plant Byproducts

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Acronyms

HS – humic substances; AFRPA - Air Force Real Property Agency; Cel – cellulose; LS – lignosulfonate; PS – pine shaving; WS – wheat straw; CC – $CaCO_3$; pyro-GCMS – pyrolysis-gas chromatography mass spectrometry; NMR – nuclear magnetic resonance; TOCSY – total correlation spectroscopy; HSQC – heteronuclear single quantum coherence spectroscopy; mHS – mobile humic substances; SOM – soil organic matter; DI – deionized; A – alanine; D – aspartate; E – glutamate; G – glycine; I – isoleucine; K – lysine; L – leucine; P – proline; Q – glutamine; R – arginine; S – serine; T – threonine; V – valine; Y – tyrosine; Py – pyrimidine; TIC – total ion chromatogram; m/z – mass to charge ratio;

Executive Summary

Humic substances (HS) are frequently the key sorption sites for metal ions in soils, especially where bioavailability is concerned. Understanding the chemical status of HS and their interactions with metal ions is critically important for long-term sustainability of any *in situ* remediation measure. However, this remains a difficult challenge. Once understood in sufficient detail, the chemical mechanism(s) of metal immobilization by soil HS may be exploited to achieve desirable remediation goals in a wide range of contaminated soils. Thus, in this proof-of-concept pilot study, we had the following two objectives:

- *Can soil humic substances be manipulated to help sequester heavy elements in soils?*
- *Can the chemical sequestration mechanism(s) be understood?*

To achieve these objectives, we investigated the chemistry of soil humification process in relation to heavy metal ion leaching by conducting soil ageing experiments with McClellan AFRPA soils. Different organic bulk materials including cellulose (Cel), lignosulfonate (LS), wheat straw (WS), pine shavings (PS) were added in combination with $CaCO_3$ (CC), along with a no amendment control (blank). The turnover of individual humic substructures was followed in separate ageing experiments by "chasing" ^{13}C and ^{15}N labels from pre-labeled HS. A combination of NMR, pyrolysis gas chromatography-mass spectrometry (Pyro-GCMS), and 3-D fluorescence techniques was employed to characterize the humified products and their ^{13}C and ^{15}N displacement. For correlation of metal mobility with humic chemistry, a broad spectrum of elements in the soil leachates was measured using ICP-MS. These new studies follow on our recent report (2) that turnover – and not the abundance – of certain HS substructures is linked to reduced metal leaching.

The results demonstrate that leaching of many metal ions from the contaminated McClellan AFRPA soil can be manipulated with a combination of $CaCO_3$ and organic amendments and by different application methods (powder versus solution form) (cf. Figs 3 & 4 for the SLS treatments). Maintenance of soil pH above neutral with $CaCO_3$ was important in reducing the leaching of transition metals and Cd but not Pb and Ba (cf. Fig. 5 and data not shown). An additive effect of organic amendment with $CaCO_3$ was observed for the reduction of metal leaching. Among the various combinations tested, the $CaCO_3$/cellulose combination was most effective in attenuating leaching of Cd and Ni (up to two orders of magnitude of reduction) whereas $CaCO_3$ plus pine shavings or wheat straw greatly reduced (more than an order of magnitude) the leaching of transition metals (cf. Figure 4). To a lesser extent, leaching of Pb, Cr, and Sr was also attenuated from all amended soils (data not shown).

The 3-D fluorescence analysis of mobile HS isolated from the unlabeled ageing experiment showed that several chromophores changed upon addition of Cd(II), with most significant changes occurring in the excitation wavelength range of 260-280 nm (corresponding to modified aromatic groups such as conjugated phenolics or fused aromatic rings). The changes of fluorescence intensity varied with Cd(II) concentrations, incubation time, and types of amendments (cf. Fig. 6). These results suggest that the fluorescence changes may indicate Cd(II) binding to HS and Cd-induced alterations in humic conformation.

To probe the detailed chemical basis for these results, a combination of NMR and Pyro-GCMS analyses were performed on isolated humic fractions and/or whole soils for the ^{13}C and ^{15}N labeled

ageing experiment. This type of analysis, which is a unique capability of our research group, can reveal the humic "chemistries" underlying the differences in metal leaching. Integrated 2-D NMR analysis (^{1}H TOCSY and ^{1}H-^{13}C HSQC) identified labeled peptidic amino acid, nucleic acid, polysaccharidic, lignic, and alkene remnants as components of the mobile HS fraction (cf. Figs. 8 & 9). In addition, the 1-D ^{13}C projection spectra of mHS from all 6 treatments (blank, CC, Cel+CC, LS+CC, PS+CC, WS+CC) revealed the changes in the relative ^{13}C abundance of various chemical groups in the amended relative to the blank mHS (cf. Fig. 10). This in turn allowed the relative turnover of these groups to be estimated. A plot of the turnover of peptidic Ala versus total Cd leached exhibited a negative correlation, with the Cel+CC treatment showing the highest turnover but the lowest Cd release (cf. Fig. 11). Such relationship has also been observed previously for a DOE Savannah River Site soil.

Analyses of the same set of mHS and corresponding whole soils by Pyro-GCMS both confirmed and complemented the NMR results. The Pyro-GCMS data verified the presence of peptidic, polysaccharidic, lignic, and alkenic substructures in both whole soils and mHS fractions (cf. Fig. 12), as is from the NMR analyses. Also, the mass spectra revealed two pools of humic substructures, one fully labeled with ^{13}C and the other with none (cf. Fig. 13). This result indicates the lack of chemical mixing or scrambling between the existing and newly humified materials. It also points to the recalcitrance of peptidic residues in soils or HS, contrary to the general dogma. The plots of substructure turnover versus total Cd leached for both whole soils (cf. Fig. 15) and mHS (Fig. 17) complemented the trend observed by NMR, i.e. a negative correlation between cellulosic turnover and Cd leaching. These plots also showed a similar correlation for lignic turnover.

Altogether, this pilot study demonstrated the utility of amendment with natural bulk materials for reducing metal leaching in contaminated military base soils. It also showed the ability to manipulate metal leaching behavior with amendment combination and different application methods. Moreover, the previously uncovered chemical mechanism relatable to the reduced metal leaching, i.e. turnover of lignocellulosic components of soil HS, also applies to the present case. A further in-depth investigation using the demonstrated approach can help answer the following questions for the purpose of sustainable and holistic metal remediation:

- Can a wide range of toxic metals be immobilized simultaneously with different combinations of chemically diverse organic and inorganic amendments?
- What is the long-term fate and bioavailability of the immobilized metals?
- Are similar chemical mechanism(s) operating in different soil types?
- Can a general set of guidelines be established for immobilizing metals in a diverse range of soils?

Answering these questions should lead to the rational design of an *in situ* bioremediation scheme that is widely applicable to contamination problems in US military bases, highly economical, sustainable, and ecologically friendly.

Objectives

The objectives of the proposed one-year exploratory work is to address the following two questions:

- *Can soil humic substances be manipulated to help sequester heavy elements in soils?*

- *Can the chemical sequestration mechanism(s) be understood?*

Background

Soil contamination of toxic heavy metals is prevalent in the U.S. military bases, which poses a serious concern to human and ecosystem health. Unlike organic contaminants, heavy metals cannot be remediated by degradation, which leaves removal, stabilization, and/or transformation of toxic to less toxic or innocuous forms as the remaining options. To successfully implement these options, fundamental understanding of metal interactions with the soil matrix is required to overcome existing technological and economic barriers and to ensure long-term sustainability.

Regardless of the specific approach, soil organic matter (SOM) is a key player in heavy metal bioremediation since it is a major component of the soil matrix that directly interacts with metal ions, thereby altering metal solubility, mobility, and bioavailability. Past reports have shown that SOM, mainly humic substances (HS), can be manipulated (via microbial and chemical modifications) to achieve desired metal availability. For example, SOM manipulation by addition of organic materials to soils shows a considerable bioremediation potential for metals as well as organic pollutants (e.g. Channel et al., 1999; Shuman, 1998). Humic amendment of sandy soils led to a lowering of Cd and Pb in the exchangeable SOM fraction and an increase in the less mobile fractions (Shuman, 1998). It is also interesting to note that leaching of metal-fulvate complexes from the surface horizons can be blocked by precipitation of these complexes with additional metal ions present in deeper horizons (3). Thus, it may be feasible to exploit SOM-metal interactions to both stabilize metal ions in surface soils and to intercept metal ion leaching in subsurface soils. However, to "bioengineer" SOM for sequestering metals requires a basic understanding of the chemical nature of SOM and its turnover pathways and kinetics in relation to metal binding, over a wide range of contaminated soils. This knowledge is also needed to assess and predict the sustainability of an *in situ* metal sequestration approach.

In our laboratories, we recently found that amending DOE Savannah River Site soil with plant byproducts of high lignin and cellulose content resulted in low Cd leaching from this soil contaminated with Cd-loaded plant roots (2). In fact, it was the faster turnover – not the abundance – of lignocellulosic materials that correlated with the reduced metal leaching. This finding prompted the questions whether this mechanism applies to *in situ* metal contaminated soils and whether different lignin byproducts can achieve a similar outcome. These questions were investigated in this report by employing several plant byproducts and a Cd-contaminated military base soil. The byproducts used included:

- Lignosulfonate (LS): a byproduct of pulpmill operation and is rich in modified lignins.
- Wheat straw (WS): a byproduct of agricultural production and is rich in lignocellulose

- Pine shavings (PS): a byproduct of building industries and is rich in lignins
- Cellulose (Cel): a commercial product of pure cellulose

These materials were chosen based on their variation in lignin content and structures as well as differences in metal leaching outcome observed by us in a previous study (2).

Materials and Methods

Soil Collection

"McClellan" soil was collected with the help of Mr. Al Calise and Mr. Don Gronstal (Air Force Real Property Agency) from a Cd-contaminated area (PRL 60) in the former McClellan Air Force Base, Sacramento, CA. The soil was regularly flooded and has additionally been contaminated with Pb, Cr, Ba, Ni, Cu, Ag, and petroleum hydrocarbons. It is relatively low in SOM presumably due to the lack of vegetation input.

Soil Processing and Ageing

The McClellan PRL 60 soil was processed to 1-2 mm size particles by hand grinding and sieving to remove rocks and non-soil materials. The % moisture (27.4%) was determined from the soil weight loss after lyophilization. The processed soil was stored in sealed bags at 4°C until use.

For ageing of unlabeled soils, 20 g each of the ground soil was amended with 1% (w/w) each of cellulose (Cel, Sigma Chemicals, St. Louis, MO), wheat straw (WS), pine shavings (PS), or 0.25-1.5% of lignosulfonate (LS) or sugared LS (SLS) (Aldrich Chemicals). Initial ageing was conducted without $CaCO_3$, which resulted in soil acidification. Subsequent ageing experiments were performed with 0.5% (w/w) $CaCO_3$ to maintain soil pH. Also included were a blank (with no amendment) and a $CaCO_3$ only treatment. $CaCO_3$ and organic amendments as powder form were mixed thoroughly with the soil before packing into a 25 ml column. The column bottom was fitted with a glass frit and a 0.45 μm cellulose membrane to prevent fine soil particles from leaching. An alternative method for LS and SLS application were done by dripping LS or SLS (40 mg/ml) dissolved in deionized water (1.25 ml) onto the soil column. For labeled experiments, 14-15 g each of the amended soil was used. The moisture of all soil columns were adjusted to about 33% before incubation at 25°C in a humified environmental chamber. The columns were irrigated weekly with deionized water. Soil leachates were collected weekly to monthly by passing 1 ml of deionized water through columns and centrifuging at 329 x g for 3 min. Ageing was conducted for 4-7 months.

^{13}C & ^{15}N Labeling of Soil

PRL 60 soil was incubated with uniformly ^{13}C-labeled glucose (3 aliquots of 2.5 g per 200 g soil) or $K^{15}NO_3$ (3 aliquots of 0.45 g per 200 g soil) for 8 months with weekly deionized water irrigation and bimonthly label supplement (see Figure 1 for setup). Parallel treatments with unlabeled glucose and KNO_3 were also performed. The soil was then equally divided and each amended with none (blank), $CaCO_3$ (0.5%) only, or $CaCO_3$ in combination with cellulose (1%), lignosulfonate (0.25%), pine shaving (1%), and wheat straw (1%).
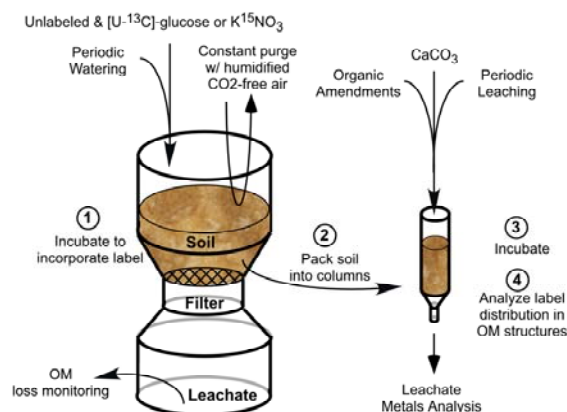
Figure 1. Setup for stable isotope labeling and ageing of soil organic matter. The filter apparatus on the left was used to prepared soil labeled with ^{13}C-U-glucose, ^{15}N-nitrate, or ^{13}C-U-glucose+^{15}N-nitrate. The upper soil compartment was aerated with humified CO_2-free air (achieved by passing air through KOH pellet). Preiodic irrigation of soil with DI water was performed to maintain soil humidity. The volume of water was controlled to minimize loss of labeled precursors via leaching. The column on the right was used for soil ageing experiments.

Leachate and soil analyses

Soil leachates were analyzed for pH and a broad spectrum of elements by ICP-MS. The elements routinely quantified included Li, Be, Na, Mg, Al, K, Ca, V, Cr, Mn, Fe, Co, Ni, Zn, Cu, Ga, As, Se, Rb, Sr, Ag, Cd, Cs, Ba, Tl, Pb, and U. The aged soils were lyophilized, ground to 250 µm size, and extracted with 0.25 M NaOH for mobile HS (mHS), acidified to remove Ca, and reextracted with 0.25 M NaOH for calcified HS (CaHS). Both HS fractions were treated with chelator Tiron to remove exchangeable cations, washed 3 times with 0.1 N HCl, and adjusted to pH 6 before lyophilization in D_2O (1).

3-D Fluorescence Analysis: The lyophilized mHS was dissolved in DI water at 0.6-1 mg/ml in the absence or presence of various Cd concentrations before fluorescence measurement using a Safire UV-Vis/fluorescence scanning microplate reader (Tecan) or a thermostatted LS55 scanning spectrofluorimeter (Perkin Elmer). The excitation and emission scan ranges were 230-490 and 270-700 nm, respectively. Contour plots of the 3-D data were made to discern spectral changes induced by Cd additions.

NMR Analysis: The mHS was dissolved in D_2O and analyzed on two Varian Inova NMR spectrometers (Varian, Inc., Palo Alto, CA) at 14.1 T and 18.8 T. The NMR experiments conducted included 1-D ^{1}H NMR, 2-D ^{1}H TOCSY (total correlation spectroscopy), 2-D ^{1}H-^{13}C HSQC (heteronuclear single quantum correlation spectroscopy), and ^{1}H-^{15}N HSQC, as described previously (4) to assign labeled substructures and to compare the label abundance. Two additional 2-D experiments (^{1}H-^{13}C HSQC-TOCSY and HCCH-TOCSY) were performed to verify the assignment of ^{13}C labeled carbons including those that were consecutively labeled. The NMR analysis of mHS was complemented with pyrolysis-GC-MS (PyGC-MS) analysis, which was also conducted for the whole soil and CaHS.

Pyrolysis-GCMS Analysis: The procedure and instruments for pyro-GCMS were analogous to that described previously (Fan et al., 2000), this time using a Frontier Labs (Koriyama, Japan) Autoshot AS1020E with PY-2020i pyrolyzer interfaced to an Agilent (Palo Alto, CA, USA) 5890 Series II gas chromatograph outfitted with a Frontier Labs UA-5 steel-clad fused silica column of 0.25mm i.d. x 30m length, 0.25µm diphenyldimethylpolysiloxane coating. This system was interfaced to an Agilent 5971A MSD mass spectrometer. Briefly stated, to analyze samples, a sample cup containing 10 mg of soil or 1mg of HS was dropped into a 550°C furnace under a He
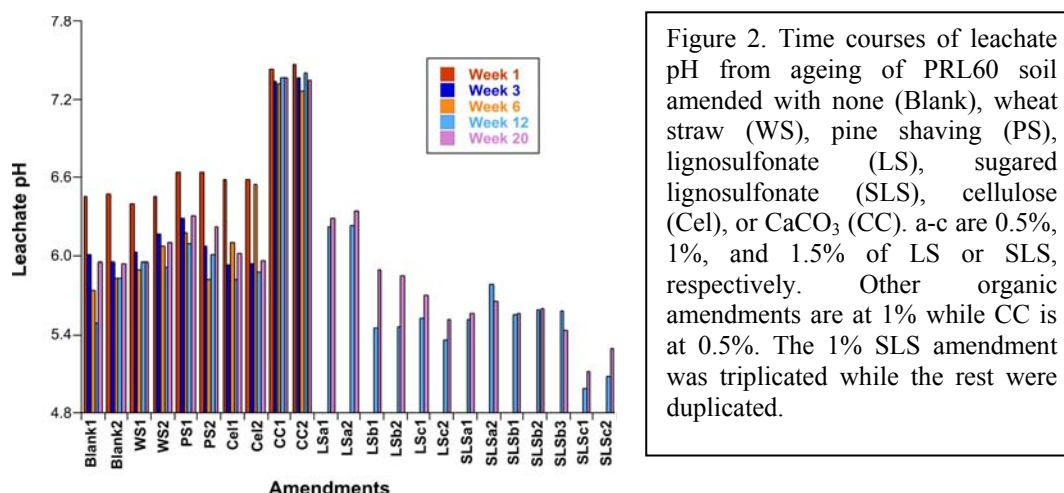
stream. This caused thermolysis of chemical bonds according to their relative strengths, which generated volatile products. The products were swept by the He stream into the column which resulted in sequential elution of the volatile products. These chemicals eluting from the column were examined by on-line mass-spectral (MS) detection; the identity of many fragments are considered traceable to the parent structural class from which they arose (5-7).

Analysis of pyro-GCMS data sets consisted of comparison of chromatographic peaks to available standards for thermolytic products, using structural matching with the aid of a 78,000-compound NIST/NIH/EPA mass spectral library. Peaks associated with a given structural class (lignins, peptides, polysaccharides) were pooled to obtain a net response which was a single numerical peak area value, as per the method of Faix et al (8). These responses were compared with pyro-GCMS analyses of purified components such as microcrystalline cellulose (Sigma), indulin AT, and bovine serum albumin (Sigma). Standard paper filters (Whatman #451) were found to be highly reproducible and was analyzed periodically to assess changes in instrument analytical response; changes were negligible (<3%) for the duration of the analyses, therefore the data warranted no instrument response correction. Data analysis for isotopic enrichment is described with the results.

Results and Accomplishments

Ageing of Unlabeled Soils

1st Ageing Experiments: Initial aging experiments performed without $CaCO_3$ amendment resulted in a drop of the leachate pH, particularly for the SLS amendment, as shown in Figure 2. The extent of the acidification for LS and SLS was also related to the % amendment and respiratory activity (as measured by CO_2 release, data not shown). These findings suggest that microbial metabolic activities were at least in part responsible for the acidification. It should also be noted that $CaCO_3$ amendment helped maintain the leachate pH above neutral during the 5-month ageing period.

Figure 2. Time courses of leachate pH from ageing of PRL60 soil amended with none (Blank), wheat straw (WS), pine shaving (PS), lignosulfonate (LS), sugared lignosulfonate (SLS), cellulose (Cel), or $CaCO_3$ (CC). a-c are 0.5%, 1%, and 1.5% of LS or SLS, respectively. Other organic amendments are at 1% while CC is at 0.5%. The 1% SLS amendment was triplicated while the rest were duplicated.

In these experiments, LS and SLS were applied as powder, which led to a significant loss of the amended material via leaching, as evident from their UV-Vis signatures in the leachates (data not shown). The leached LS and SLS were accompanied by a much enhanced metal leaching (relative to the blank treatment) as illustrated in Figure 3 for Pb (cf. Week 8 data). A similar trend was also evident for Cr, V, Cu, and Ag. This increased mobilization of metals correlated with the amount of LS or SLS applied, which may reflect the binding capacity of lignosulfonates for these metals. It may also be related to the soil acidification observed for the LS and SLS treatments (Figure 2). A similar observation was also made for a parallel set of ageing experiments conducted with another Cd-contaminated McClellan soil (A6) (data not shown). Based on these results, subsequent ageing experiments were conducted by applying a reduced amount of LS and SLS (0.25%, w/w) and as solution to enhance their adsorption to the mineral matrix. In addition, all organic amendments were applied together with 0.5% $CaCO_3$ to help maintain a neutral pH.

2nd Ageing Experiments: The inclusion of $CaCO_3$ along with the organic amendments prevented the soil acidification, with all leachate pH's remained above neutral throughout the 5-month ageing period (data not shown). Applying LS and SLS in solution forms also minimized their initial leaching from the soil column (data not shown). These measures had a profound impact on the metal leaching profiles, as shown in Figure 4, where the total amounts of metals leached over 5 months were plotted as a function of the treatment. It is clear that the excessive leaching of Pb, Cr, and Cu was eliminated by the modified approach (cf. Figs 3 and 4). It is also evident that $CaCO_3$

(CC) treatment alone dramatically reduced the amount of transition metals, Cd and Cr leached from the soil. This could be related to the maintenance of soil pH near neutral. However, $CaCO_3$ did not prevent Pb leaching while enhancing the release of Ba (data not shown). Also noted was an additive effect of cellulose (Cel) amendment on reducing the leaching of the transition metals and Cd.
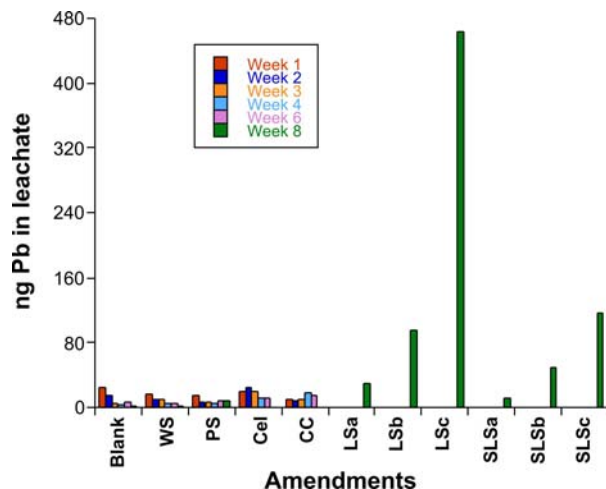

Figure 3. The Pb leaching time courses for the ageing experiments described in Figure 2. Leachate collection started on Week 1 for the Blank, WS, PS, Cel, and CC treatments while that for the LS and SLS treatments began on Week 8 to allow the amendment to interact with the soil matrix.
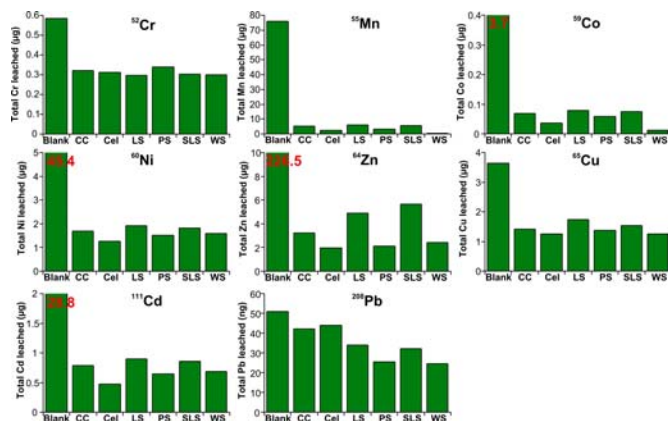

Figure 4. Total metal profiles of leachates from the 2nd ageing experiment. The values for Co, Ni, Zn, and Cd were plotted off-scale and were listed in red. All acronyms are as in Figure 1.

Ageing of ^{13}C and [15]N Labeled Soils

To investigate the chemical mechanism of the amendment effect, the PRL 60 soil was labeled with ^{13}C, ^{15}N, or [13]C+[15]N by incubating the soil with ^{13}C-glucose, ^{15}N-nitrate, or ^{13}C-glucose+[15]N-nitrate for 8 months. The prelabeled soil was then amended with $CaCO_3$ plus unlabeled organic matters and incubated for 7 more months. The 1st leachate was collected after 1 month of incubation and a total of 6 leachates were obtained.

3rd Ageing Experiment: Again, the leachate pH in this labeled experiment remained above or near neutral for all but the blank treatment which acidified to as low as pH 5.3 after 7 months. The time course changes of transition metals, Cd, and Pb for the ^{13}C labeled experiment are shown in Figure 5. Other parallel labeled experiments exhibited a qualitatively similar trend as that in Figure 5. The blank treatment with no amendment contained the highest amounts of metals in all leachate

collections, except for Mn. In contrast, the cellulose treatment had the lowest metal content in most leachate collections. The leachate time course for Pb differed from those of the other metals, showing a large increase after the 4th collection for all treatments. This increase was most pronounced for the $CaCO_3$ only treatment, which is consistent with the observation for the unlabeled 2nd ageing experiment. Displacement of bound Pb by Ca may underlie this behavior. In addition, the Pb time courses here differed from those of the 2nd ageing experiment (Figs. 4 and data not shown) which did not show a "breakthrough" of Pb. This difference may be to do with the longer length of incubation (7+8 months) and/or added influence of glucose and nitrate.
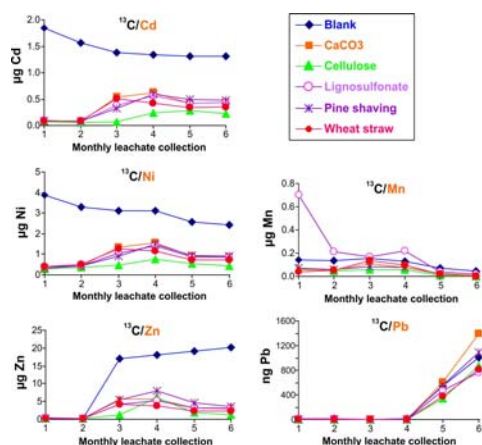

Figure 5. Time courses of metal leaching for the 3rd (^{13}C-labeled) ageing experiment. Each treatment was duplicated and all data points were an average of the duplicates.

3-D Fluorescence Analysis of Humified Products

The mobile HS (mHS) isolated from the 2nd ageing experiment was subjected to 3-D fluorescence analysis both in the absence and presence of $CdSO_4$. Distinct changes in the 3-D spectrum in all mHS samples were observed upon addition of Cd, where emission spectral changes were most significant with excitation wavelengths of 260 to 280 nm. Example emission spectra of two mHS preparations as a function of Cd concentrations are shown in Figure 6. Decreases in fluorescence intensity were evident in both sets of spectra with maximal changes centered about 400 nm. The intensity changes followed a non-linear function of the added Cd concentrations, reaching a plateau value at 0.2 mM Cd. A further decrease in intensity was noted after 3 days of mHS interaction with Cd, as illustrated for the WS treated sample. Altogether, these results suggest that the Cd-induced fluorescence changes of mHS resulted from Cd binding and that this binding may lead to a rapid and a slower conformational change in the HS.

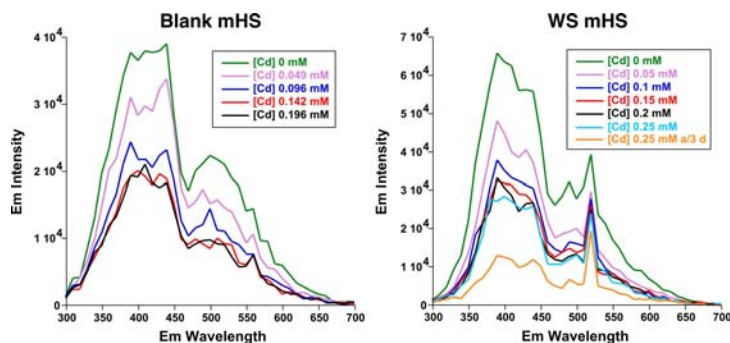Figure 6. Fluorescence emission spectra of blank and WS-treated mHS isolated from the 2nd Ageing experiment. The excitation wavelengths for the blank and WS spectra were 280 and 260 nm, respectively. $CdSO_4$ was added sequentially to mHS to the specified concentrations. For the WS-treated mHS, spectrum was also taken after 3 days of incubation with Cd at room temperature.

NMR Analysis of Humified Products

^{1}H NMR of Mobile HS (mHS): The ^{1}H NMR spectra were acquired at 18.8 T to maximize the spectral resolution. Figure 7 illustrates the 1-D spectra for all 6 treatments of the ^{13}C-prelabeled soil. All spectra were scaled for direct comparison of peak intensity, which reflected the abundance of individual substructures of mHS. The assignment of these substructures was made based on the 2-D ^{1}H TOCSY (cf. Figure 8) and ^{1}H-^{13}C HSQC spectra (cf. Figure 9) described below. As expected, the aromatic/phenolic structures in the mHS of LS, PS, and WS were more abundant relative to those in the mHS of blank, CC, and Cel, since the former group of amendments is rich in lignins or phenolics. The abundance of peptidic alanine (A), valine (V), leucine (L), and isoleucine (I) was somewhat attenuated in the mHS of Cel and WS treatments, which may be related to their faster turnover (see result below). It is also interesting to note the substantial increase in the intensity of a sharp peak near 0 ppm for the LS and PS treatments. This peak most likely arose from an organosilicon group, which has been observed in the ^{1}H NMR spectra of other HS preparations (1).
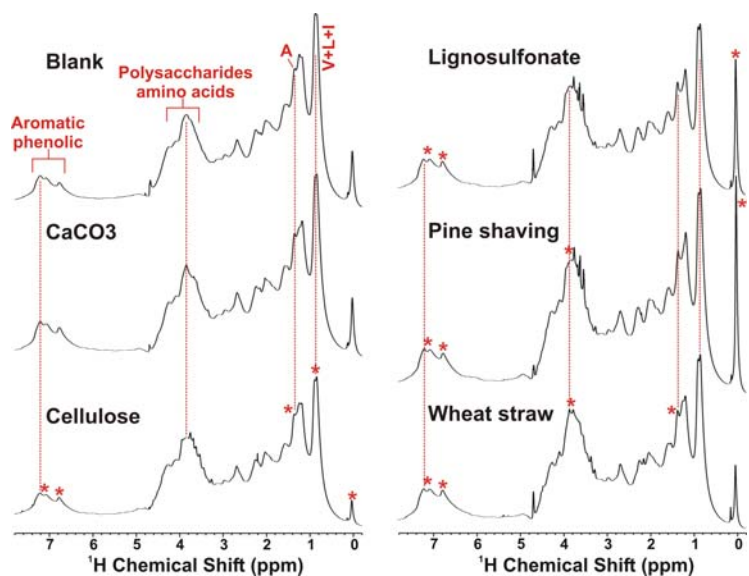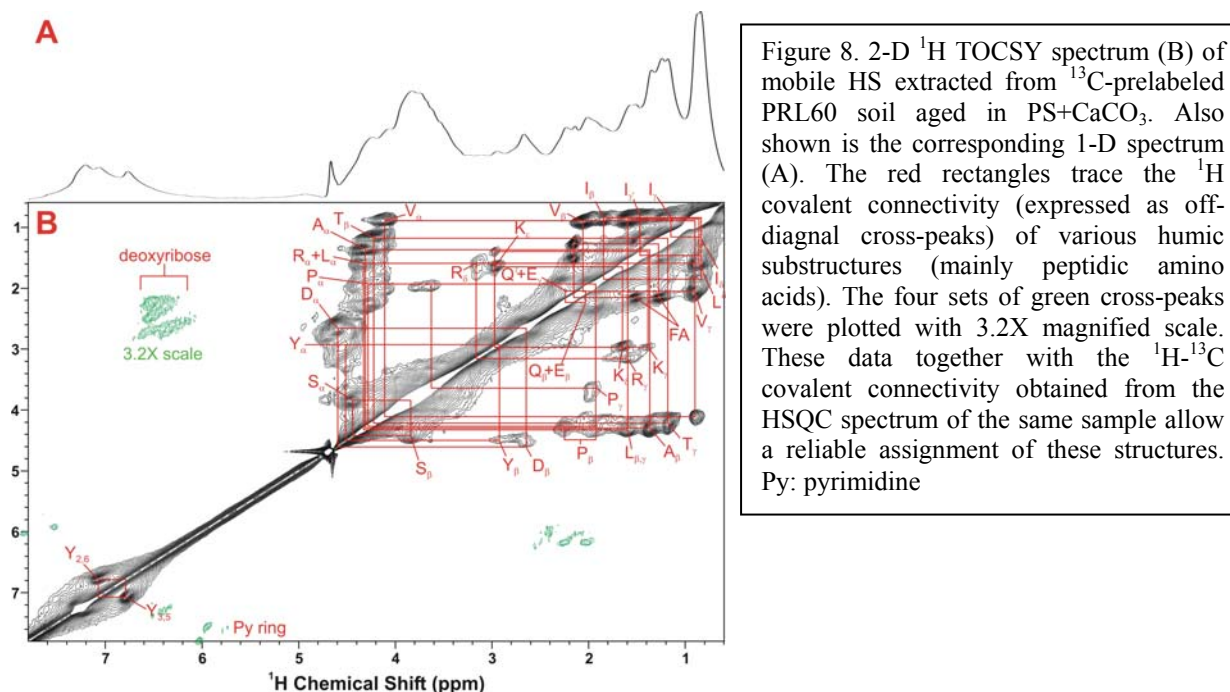

Figure 7. 1-D ^{1}H NMR spectra of mobile HS extracted from aged PRL60 soil prelabeled with ^{13}C-glucose. The NMR spectra were acquired at 18.8 T and 30°C. The peak intensity of all spectra was normalized to that of the cellulose treatment for comparison. The * denotes a difference in ^{1}H abundance for the amended HS from that for the blank HS, e.g. an increase in the aromatic/phenolic noted for the LS, PS, and WS HS while a decrease evident for the Cel HS.

To obtain detailed chemical assignment for the 1-D ^{1}H NMR spectra, 2-D TOCSY spectra were acquired on the same set of mHS samples. Figure 8 illustrates the TOCSY spectrum of the mHS for the PS treatment. The ^{1}H covalent linkages, as represented by off-diagonal cross-peaks, were traced by the rectangular boxes. This, together with the chemical shift information, allowed the assignment of various peptidic amino acid residues and structural components of nucleic acids such as the deoxyribose and pyrimidine ring residues. As noted previously (1, 4), the TOCSY spectrum was dominated by the covalent linkages (cross-peaks) of peptidic amino acids while those for the polysaccharides were absent. Based on the chemical shift and py-GC-MS data (see below), polysaccharidic structures were present in all mHS samples. This lack of covalent linkage signatures may reflect a restricted molecular environment for the saccharides in the HS.

2-D ^{1}H-^{13}C NMR of Mobile HS (mHS): To verify and complement the ^{1}H NMR assignment, 2-D ^{1}H-^{13}C HSQC experiments were performed on the same set of mHS samples as in Fig. 7. Two example HSQC spectra are shown in Figure 9, where the spectra of the blank and Cel-CC-amended mHS are compared. The [1]H-^{13}C covalent linkages (expressed as cross-peaks) confirmed

the peptidic assignment made in Figure 8 while revealing the assignment for polysaccharides, alkenes, and methoxy groups from lignaceous structures. In addition, the peak intensity in the 1-D ^{13}C projection spectra indicates the relative abundance of ^{13}C label in each chemical group. By comparing spectra A and C, it is clear that most, if not all, of the chemical groups in the Cel-CC amended mHS were much lower in ^{13}C abundance that those in the blank treatment. This finding suggests that the preexisting ^{13}C labels in the former HS was "chased" out faster than those in the latter HS, i.e. the organic matter in the Cel-CC treatment was turned over faster than that in the blank treatment.

Figure 8. 2-D ^{1}H TOCSY spectrum (B) of mobile HS extracted from ^{13}C-prelabeled PRL60 soil aged in PS+CaCO$_3$. Also shown is the corresponding 1-D spectrum (A). The red rectangles trace the ^{1}H covalent connectivity (expressed as off-diagnal cross-peaks) of various humic substructures (mainly peptidic amino acids). The four sets of green cross-peaks were plotted with 3.2X magnified scale. These data together with the [1]H-^{13}C covalent connectivity obtained from the HSQC spectrum of the same sample allow a reliable assignment of these structures. Py: pyrimidine

The mHS turnover was in turn related to the total amount of Cd and Pb leached from the soil, as shown in Figure 11, where the relative loss of peptic ^{13}C-Ala from mHS (amended minus blank) was used to indicate the mHS turnover. A negative correlation between the mHS turnover and the total amount of leached Cd and Pb was observed, e.g. cellulose addition showed the highest mHS turnover but the lowest extent of Cd and Pb leaching. It should be noted that the CaCO$_3$ only treatment had a similar mHS turnover as the blank but exhibited much less Cd leaching than the blank. The increased Cd leaching could be attributed to the acidification of the blank soil. Soil pH appeared to have less an effect on Pb leaching, while mHS turnover was more effective. The inverse correlation of humic turnover with Cd leaching has also been observed for a different soil (from DOE SRS site) previously (2). Further work with other contaminated soils is needed to determine whether this mechanism is generally applicable.
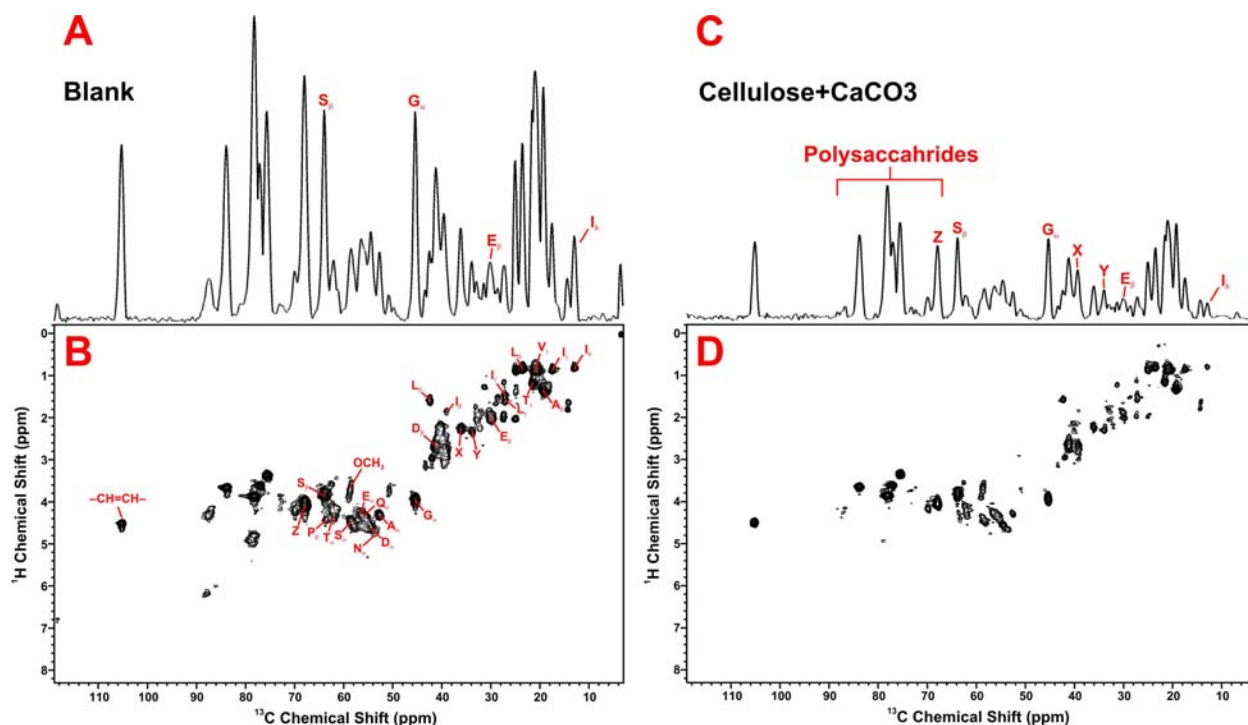
Figure 9. 2-D ^{13}C-^{1}H HSQC NMR spectra (B,D) are shown with the corresponding 1-D ^{13}C projection spectra (A,C) of mobile HS (mHS) isolated from ^{13}C labeled PRL60 soils amended with none (blank) or with cellulose+CaCO$_3$ (Ce-CC). The HSQC spectra allowed ^{13}C-enriched peptidic substructures of mHS to be discerned while the peak intensity of the 1-D projections compared the ^{13}C abundance between the two treatments. Relative to the blank, the Ce-CC treatment shows an overall large decrease in the ^{13}C abundance of various peptidic residues, which indicate a faster turnover of these carbons in the latter treatment. A differential turnover of peptidic glutamate (Eβ), isoleucine (Iδ), glycine (Gβ), and serine (Sβ) relative to other humic carbons was also observed for the Ce-CC-treated soil (cf. Ä & B). X, Y, Z denotes unassigned peaks. Polysaccarhidic and alkenic peaks were tentatively assigned based on the ^{1}H and ^{13}C chemical shifts.
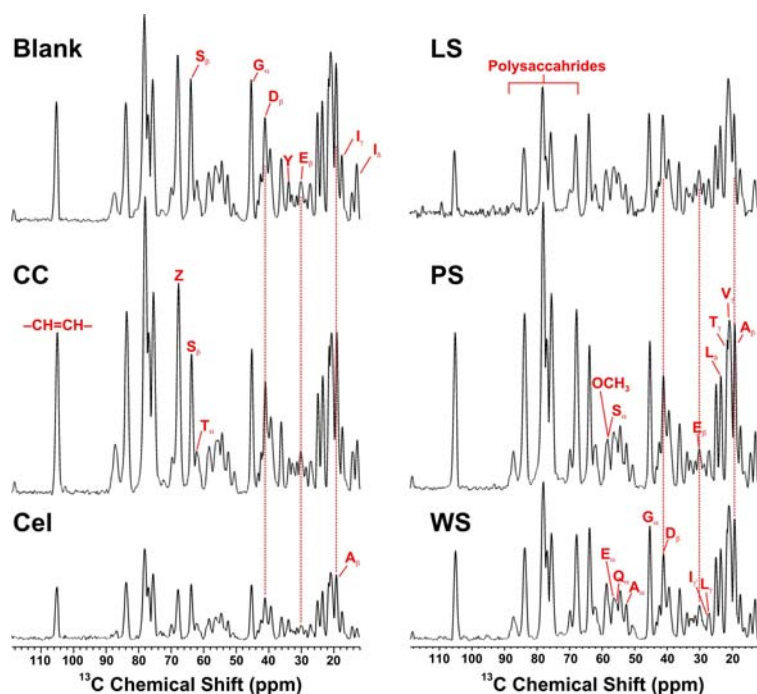

Figure 10. ^{13}C HSQC projection spectra of mobile HS extracted from ^{13}C-prelabeled PRL60 soil aged in various amendments. Acronyms are as defined in Materials & Methods. Cel, LS, PS, and WS amendments also contained CaCO$_3$ (CC). The peak intensity reflects ^{13}C abundance of various humic substructures, of which many were assigned to peptidic amino acids (cf. Fig. 9). The OCH$_3$ group assigned is likely to arise from lignaceous structures.
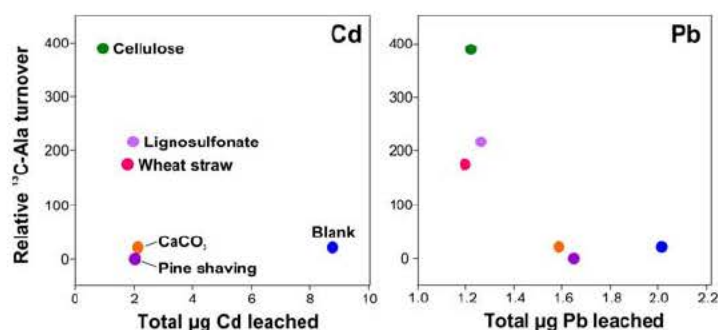
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Figure 11. mHS turnover versus total Cd leached from ^{13}C-labeled PRL60 soils aged as in Figure 9. mHS turnover is represented by the reduction in ^{13}C peak intensity of the peptidic alanine (A_β) for the amended mHS relative to the blank mHS, as measured from Fig. 10.

Pyro-GCMS Studies of HS Turnover and Abundance

The peptidic alanine turnover described above may reflect the fate of selected substructure(s) of mHS, which could differ from the fate of other parts of mHS. To examine the general turnover property of organic matter in the 3rd ageing experiment, pyro-GCMS analysis was employed.

As with NMR, pyro-GCMS is capable of detecting ^{13}C and ^{15}N labels in various structure components of SOM, in particular HS. Thus, the humification kinetics of different OM structure components may be discerned by these techniques. For its part, pyro-GCMS analysis typically yields hundreds of thermolytic-mass fragments that are derived from various components of SOM including lignaceous, cellulosic, proteinaceous, polysaccharidic, long-chain alkanes, etc. The sheer complexity of the data yields a richness of pattern unique for a given soil or OM. Figure 12 describes the pyro-GCMS process. Such "chemical fingerprinting" capability offers an excellent opportunity for a comprehensive chemical profiling of SOM and its isolated HS, from which isotopic enrichment of a given chemical group can also be quantified. Despite its capability of producing extremely detailed data on HS, chemical reactions can occur during thermolysis, which precludes reliance on pyro-GCMS alone for structure analysis of complex HS. When coupled with NMR, which provides structure information on intact SOM, a detailed, yet more reliable structure characterization of HS can be achieved (1).
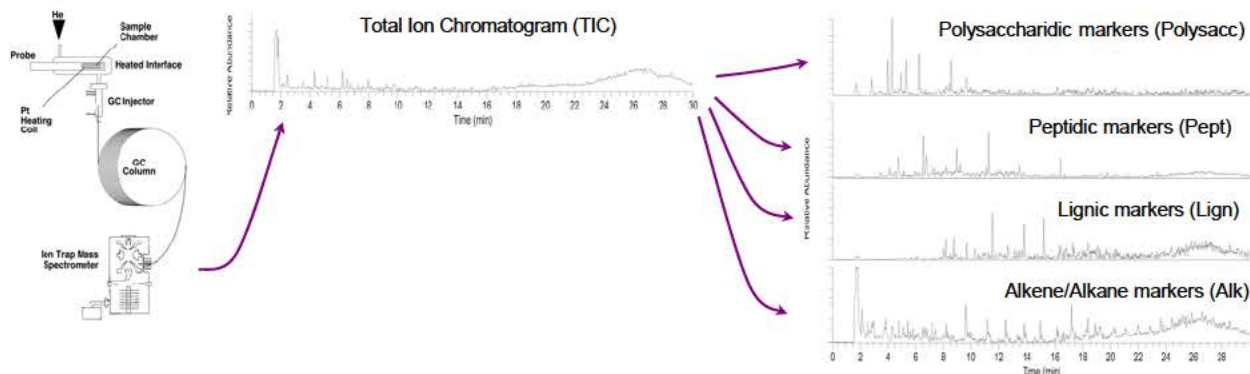

Figure 12. Brief description of the pyro-GCMS analytical process. Soil or HS samples are placed in a He-flushed sample chamber, which is then ballistically heated to 550°C. Thermolyzed fragments enter a GC where they are separated and analyzed by an MS. The result is a TIC (above). MS ion data indicative of HS sub-structures, such as peptides, are extracted from the TIC (panels at right) and quantified. This data analysis step is repeated for each appropriate stable isotope ion; in the present case, there were 61 such data analysis cycles per sample.

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This specific strategy of relating detailed HS structures to metal leaching has been successful in soils from the DOE Savannah River Site (2). In that study – as with this study - we used a combination of NMR and pyro-GCMS analyses to determine the residual ^{13}C labeling of HS substructures, which revealed that turnover—not the abundance *per se*—of polysaccharidic and lignic HS substructures was associated with reduced leaching of Cd, Ni, and Cs.

Pyro-GCMS Analysis of Whole Soils: To gain an overview profile of the organic matter, whole soil was first analyzed by pyro-GCMS. The main advantage of directly analyzing whole soils is minimal sample preparation, which can help avoid unexpected chemical alterations during HS extraction. However, there are disadvantages, of which the major ones are the unavoidable inclusion of live microbial biomass, and possible alteration of result because of cross-reaction with inorganic materials. These disadvantages are largely alleviated by analyzing the extracted HS (as with the NMR), which is described in the section following this one.

Pyro-GCMS analysis of the whole soil revealed several interesting results. First, the incorporation of isotope in both ^{13}C and ^{15}N experiments were clear in most peaks by pyro-GCMS analysis, indicating extensive incorporation of the isotopes into a wide range of SOM constituents. Secondly, examination of a peak (methyl indole) representing the peptide bond structure showed two distinct pools of peptidic materials for the ^{13}C experiment, those with all carbons labeled and those with no carbons labeled. The interpretation of the pyro-GCMS pattern is explained in Figure 13. This indicates that partial carbon incorporation into peptide bonds was negligible under these conditions, such that incorporation of glucose carbons into peptides was an all-or-none process. This also indicates that, after many weeks, there remains a pool of peptidic material that does not turn over in this system. This is consistent with our earlier finding of a considerable pool of peptidic material remaining in natural soil humics (1, 4), contrary to the "old school" assumptions, based on no direct evidence, that peptidic material will be rapidly turned over in soil.
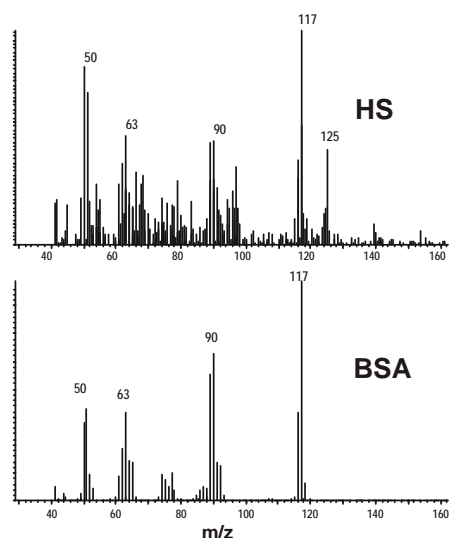

Figure 13. An example of how isotopic data is examined in pyro-GCMS. Illustrated are pyro-GCMS outputs showing pattern of labeled peptidic groups in the soil incubated with ^{13}C glucose (upper panel). Pyro-GCMS thermolyzes peptidic groups to form indole (C_8NH_{10}) with expected all-^{12}C ion at mass-to-charge ratio (m/z) 117, and the smaller natural abundance ^{13}C ion at m/z 118. Depending on the number of carbons labeled, a distribution of peaks from m/z 117 –> 125 can be expected to be seen for this peptidic marker, with m/z 125 representing a fully ^{13}C-labeled structure. An example of non-labeled standard peptidic material, bovine serum albumin (BSA), is shown in the bottom panel. In the upper panel, there appears to be primarily two major pools of soil peptidic structures: non-labeled (m/z 117), and fully labeled (m/z 125). The non-labeled pool, persisting after 34 weeks of incubation with ^{13}C glucose, apparently represents a "recalcitrant" pool of peptidic material in soil, consistent with previous findings (1).

Overall, the pyro-GCMS analysis revealed a plethora of detailed changes in the organic matter over the course of the experiment. Figure 14 shows the trends that were observed between % Turnover and Abundance across the various treatments (Blank, CaCO$_3$, WS, PS, Cel, and LS). It is interesting to note that the turnover of soil lignic structures varied widely while their abundance

changed little across different treatments. The opposite was observed for peptidic and polysaccharidic structures.
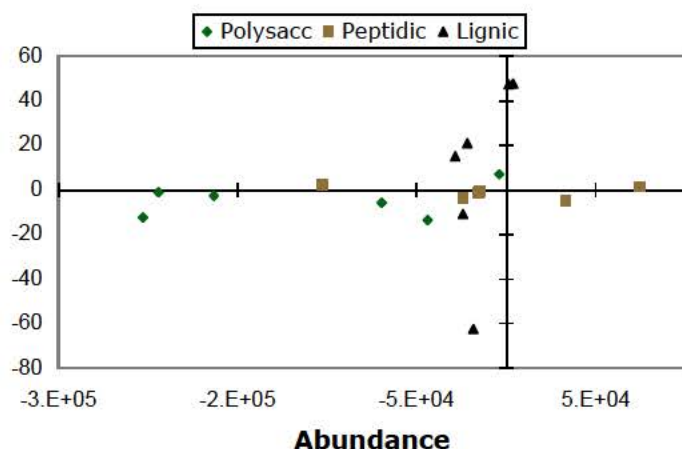

Figure 14. Relationship between % turnover and abundance in whole soils as measured by pyro-GCMS. As shown here, the different treatments (Blank, $CaCO_3$, WS, PS, Cel, and LS) with natural bulk materials successfully created a wide range of responses in the different 'chemistries" of HS. Each datum is a treatment (labels omitted for clarity). In summary, the Polysaccharidic (green diamonds) and Peptidic (brown square) structures varied primarily in Abundance among treatments, while Lignic (black triangle) structures varied mostly in the % Turnover axis.

The pyro-GCMS data was further examined by plotting Turnover and Abundance of the three constituents against the total amount of Cd leached during the experiment. Figure 15 shows the two parameters that appeared to relate to Cd leaching. Taken together, the NMR and pyro-GCMS evidence suggests that, for peptidic structures, both Turnover and Abundance may be important for reduced leaching. Unlike NMR, differences in protein Turnover were too small to be measured effectively by pyro-GCMS. Therefore, pyro-GCMS was not able to immediately confirm the NMR findings; instead the technique contributed complementary information.
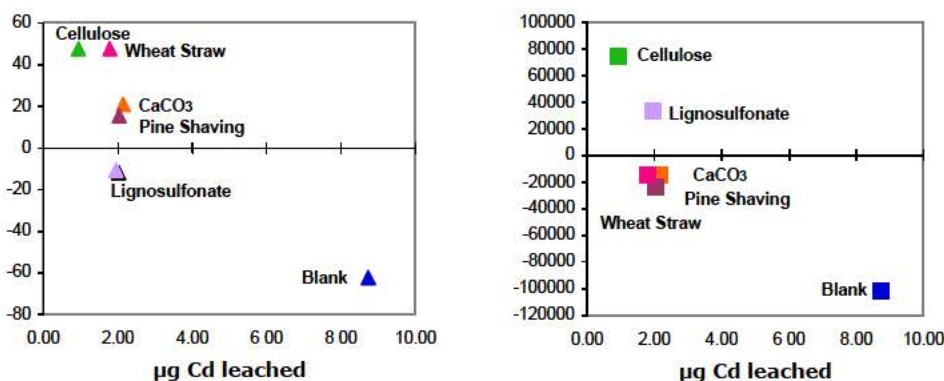

Figure 15. Relationships between Turnover or Abundance vs. Cd leaching, as measured by pyro-GCMS analysis of whole soil. Left panel: Pyro-GCMS revealed that Lignic structures Turnover displayed a possible sigmoidal function with Cd leaching. As with the NMR data, the cellulose and blank treatments showed clear opposing trends. Right panel: There was also a relationship of Cd leaching with the changes in Peptidic Abundance over the course of the experiment. It is possible that this is also a sigmoidal function.

Pyro-GCMS Analysis of mHS: To compare with the NMR results of Turnover, and to avoid the potential problems with analyzing whole soils by pyro-GCMS (see preceding section), the very same mHS samples analyzed by NMR were also subjected to pyro-GCMS analysis. Figure 16 reveals that the relationships between Turnover and Abundance seen from direct analysis of whole

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soils (Figure 14) did not hold for the lignic or peptidic structures of mHS. Contribution of organic matter other than mHS to whole soils could underlie this difference.
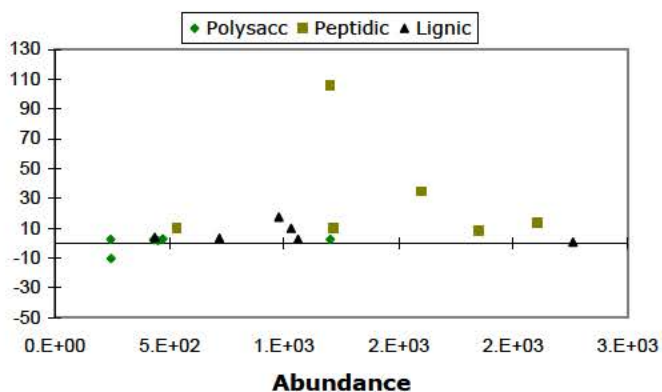

Figure 16. Relationship between turnover and abundance in mHS as measured by pyro-GCMS. This figure is comparable to Fig. 14, except that mHS was analyzed. Each datum is a treatment (labels omitted for clarity). In summary, the relationship between turnover and abundance of the lignic and peptidic structures observed for whole soils (Fig. 14) did not hold for mHS - Polysaccharidic (green diamonds), Peptidic (brown square), and Lignic (black triangle).

Once again, we compared both Turnover and Abundance across all treatments for each of the mHS constituents (polysaccharidic, peptidic, lignic), with Cd leaching. Figure 17 shows the correlation of Turnover of lignic and peptidic constituents in mHS with Cd leaching. None of the others comparison showed a trend, e.g. the peptidic abundance relation to Cd leaching (Figure 14) did not hold. As mentioned above, this difference in trend between whole soils and mHS could be due to the complication from other organic materials such as microbial biomass in whole soils.

The lignic Turnover relationship to Cd leaching (Figure 17, left) appeared to be similar to that observed from pyro-GCMS analysis of whole soils (Figure 15), while the peptidic Turnover relationship (Figure 17, right) bore some similarity to the results from NMR analyses (Figure 11). It should be noted that the NMR turnover analysis was based on the specific alanine residue while the pyro-GCMS analysis was derived from the peptide structures of many different residues. The similarity or difference between the two analyses could reflect a similar or differential turnover between alanine and other amino acid residues, as noted in Fig. 10.
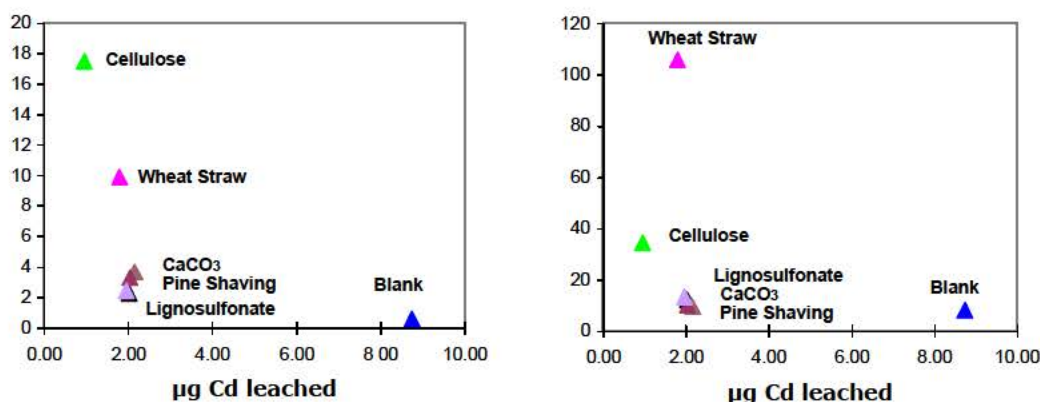
Figure 17. Relationships between Turnover of mHS vs. Cd leaching, as measured by pyro-GCMS. Left panel: Pyro-GCMS revealed that Lignic structures Turnover related to Cd leaching, a relationship similar to that from analysis of whole soil. Right panel: Also observed was a relationship of Cd leaching with the changes in Peptidic Turnover over the course of the experiment, a result comparable to that from NMR analyses (Fig. 11).

Conclusion

This proof-of-concept pilot study demonstrated the utility of amendment with natural bulk materials for reducing metal leaching in contaminated military base soils. More importantly, it showed that the manipulation of the metal leaching behavior with amendment was due to turnover of lignocellulosic components of soil humic substances.

The findings are consistent with past literature regarding the role of SOM as metal sink while contrast to the general belief in the degradability of peptides in soil humics. In addition, the present study brings up several issues that are useful to explore in the context of sustainable and holistic metal remediation:

- Can a wide range of toxic metals be immobilized with different combinations of chemically diverse organic and inorganic amendments?
- What is the long-term fate and bioavailability of the immobilized metals?
- Are similar chemical mechanism(s) operating in different soil types?
- Can a general set of guidelines be established for immobilizing metals in a diverse range of soils?

The fulfillment of these questions can lead to an *in situ* bioremediation scheme that has the following advantages:

- readily deployable in most, if not all, US military bases
- highly economical with low startup and maintenance costs
- long-term sustainability
- less, if not the least, perturbing to soil ecosystems

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Appendices (data, list of technical publications)

"Stable-isotope tracer studies of soil humification and immobilization of heavy metals", Teresa W.-M. Fan, Teresa A. Cassel, Peter G. Green, Andrew N. Lane, and Richard M. Higashi, DOE/SERDP symposium, Washington, DC, Nov. 2003.

"Soil amendment and humification for immobilization of heavy metals", Teresa W.-M. Fan, Vennila Arumugum,, Elizabeth Field, Peter G. Green, and Richard M. Higashi, SERDP symposium, Washington, DC, Nov., 2004.

"Stable-isotope tracer studies of soil humification and immobilization of heavy metals", manuscript in preparation.
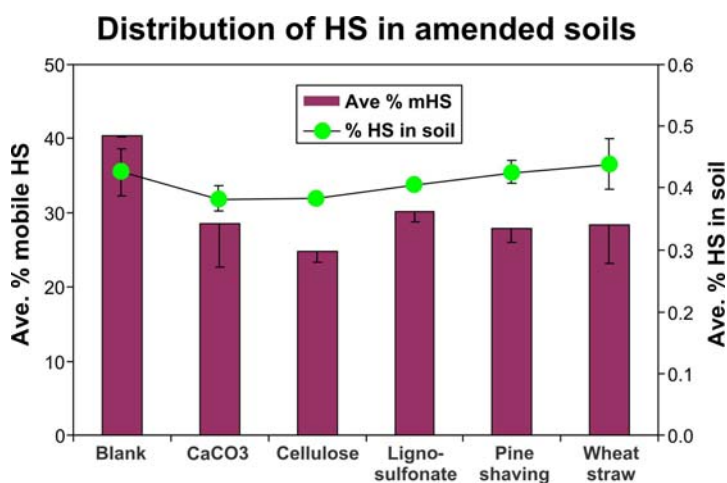
Figure 18. Distribution of humic substances (HS) in ^{13}C-labeled McClellan soils aged in none (blank) or CaCO$_3$ with a combination of different organic amendments. % mobile HS (mHS) was calculated from mHS dry weight against the total dry weight of mHS and calcified HS (CaHS); the total HS dry weight was divided by the dry weight of the soil to obtain % HS in soil. Each data point was an average of two replicate samples. The % HS in soil remained constant among treatments but the % mHS was lower in the amended relative to the non-amended samples, with cellulose+CaCO$_3$ showing the lowest % mHS (or the highest % CaHS). A redistribution of mHS into the CaHS fraction induced by organic and CaCO$_3$ supplements may account for this result.

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